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OxyScan™ cGPx-340 Assay System

Automated Determination of Cellular Glutathione Peroxidase

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Catalog Number 51017
READ ENTIRE INSERT BEFORE BEGINNING ASSAYS!

SUMMARY AND EXPLANATION OF THE TEST ANALYTE

Cellular glutathione peroxidase (c-GPx, EC 1.11.1.9) is a member of a family of GPx enzymes whose function is to detoxify peroxides in the cell.1 Because peroxides can decompose to form highly reactive radicals, the GPx enzymes play a critical role in protecting the cell from free radical damage, particularly lipid peroxidation. The GPx enzymes catalyze the reduction of H_2O_2 to water and organic peroxides (R-O-O-H) to the corresponding stable alcohols (R-O-H) using glutathione (GSH) as a source of reducing equivalents:

$$\text{R-O-O-H} + 2\text{GSH} \xrightarrow{\text{GPx}} \text{R-O-H} + \text{GSSG} + H_2O$$

With the exception of phospholipid-hydroperoxide GPx, a monomer, all of the GPx enzymes are comprised of 4 identical subunits (monomer Mr 22-23 kDa). Each subunit contains a molecule of selenocysteine in the enzyme active site. The selenocysteine is thought to participate directly in electron donation to the peroxide substrate and become oxidized in the process. The enzyme then uses glutathione as an electron donor to regenerate the reduced form of the selenocysteine1. The GPx enzymes accept a wide variety of organic peroxides as substrates. However, with the exception of phospholipid hydroperoxide GPx and perhaps pl·GPx, the enzymes exhibit a strong preference for glutathione as a source of reducing equivalents. Phospholipid-hydroperoxide GPx (Mr 19 kDa) is the only enzyme with significant activity on esterified phospholipids and cholesterol in membranes.

PRINCIPLES OF THE PROCEDURE

The GPx-340™ assay is an indirect measure of the activity of c-GPx2. Oxidized glutathione (GSSG), produced upon reduction of an organic peroxide by c-GPx, is recycled to its reduced state by the enzyme glutathione reductase (GR):

$$\text{R-O-O-H} + 2\text{GSH} \xrightarrow{\text{c-GPx}} \text{R-O-H} + \text{GSSG} + H_2O$$

$$\text{GSSG} + \text{NADPH} + H^+ \xrightarrow{\text{GR}} 2\text{GSH} + \text{NADP}^+$$

The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340 nm (A_{340}) providing a spectrophotometric means for monitoring GPx enzyme activity. The molar extinction coefficient for NADPH is 6220 M-1 cm-1 at 340 nm. To assay c-GPx, a cell or tissue homogenate is added to a solution containing glutathione, glutathione reductase, and NADPH. The enzyme reaction is initiated by adding the substrate, tert-butyl

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hydroperoxide, and the A_{340} is recorded. The rate of decrease in the A_{340} is directly proportional to the GPx activity in the sample.

REAGENTS
The reagent set is sufficient for 300 determinations (100 individual samples in triplicate) and consists of the following components:

NADPH Reagent	24 μmol glutathione, 12 U glutathione reductase, 4.8 μmol §-nicotinamide-adenine dinucleotide phosphate (reduced), 5 lyophilized vials - each equivalent to one carousel of assays.
NADPH Diluent	0.05 M Tris-HCl, 5 mM EDTA, pH 7.6, 2 bottles of 25 mL each.
tert-Butyl Hydroperoxide	*t*-BOOH 70% in water, 2.0 mL.
Sample Diluent	0.05 M Tris-HCl, 5mM EDTA, 1 mg/mL bovine IgG, pH 7.6, 120mL.

Two 30 mL Nalgene bottles are provided for dilution of the *t*-butyl hydroperoxide and are labeled, "STEP 1 DILUTION" and "*t*-BOOH WORKING DILUTION."

Warnings and Precautions
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For *In Vitro* Use Only.
The ***tert*-Butyl Hydroperoxide** is a strong oxidizer. Keep away from flammables. In case of contact with skin or eyes, rinse immediately with plenty of water. Seek medical advice.

Reagent Storage and Stability
The NADPH Reagent should be stored at ≤-20°C. Store all other reagents at 2-8°C in the dark. Unopened reagents are stable until expiration date printed on the label.

Reagent Preparation

- **NADPH Reagent:** Add 7.5 mL of NADPH Diluent to a NADPH Reagent vial (one per carousel). Use for one carousel run, dispose of any remaining reagent. DO NOT FREEZE THE RECONSTITUTED REAGENT.
- ***tert*-Butyl Hydroperoxide:** Dilute 1/10,000 in deionized water. The Working Dilution (0.007% *t*-BOOH) may be kept at room temperature for 8 hours. It should be made fresh each day.

  Example of dilution protocol:
  Step 1 Dilution (1/100 dilution of *t*-BOOH concentrate):

  0.05 mL *t*-BOOH concentrate + 4.95 mL deionized water

  Working Dilution (1/10,000 dilution of t-BOOH concentrate, 1/100 dilution of Step 1 Dilution):

  0.2 mL first dilution + 19.8 mL deionized water

SAMPLE COLLECTION GUIDELINES
Erythrocyte Lysates:

1. Draw blood into an anticoagulant tube and mix by inversion.
2. Centrifuge at least 500 mL whole blood at 2500 x g for 5 minutes at 4°C.

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3. Discard plasma supernatant, wash cells 3 times in ice cold 0.9% NaCl.
4. Resuspend the packed cells in 4 volumes of ice cold deionized water, vortex well.
5. Centrifuge at 10,000 x g for 5 minutes at 4°C.
6. Collect the supernatant for assay.
7. Dilute supernatant 1/100 with Sample Diluent provided.

Tissue Homogenates:

1. Wash tissue in 0.9% NaCl solution, blot dry.
2. Homogenize tissue in cold Sample Diluent at a ratio of 1 to 6 (w/v).
3. Centrifuge homogenate at 3000 x g for 10 minutes at 4°C.
4. Collect upper aqueous layer for assay.

Storage and Handling of Sample

- **Erythrocytes:** If not lysed immediately, packed cell pellets may be frozen at -70°C.
- **Tissue:** If not homogenized immediately, wrap tissue in aluminum foil and flash-freeze. Store at -70°C.
- **Supernatant from any Sample:** If not assayed immediately following precipitation, supernatant should be frozen at -70°C.

PROCEDURE
Instrument
OxyScan™ Instrument, Catalog Number 51101

Materials Required But Not Provided

- OxyScan™ Cuvettes, Catalog Number 81003
- OxyScan™ GPx-340 Calibrator Kit, Catalog Number 52017
- OxyScan™ 340 nm Photocalibrator, Catalog Number 51117
- Microcentrifuge tubes
    - Centrifuge
    - Pipettes with disposable tips
    - Vortex mixer
    - 0.9% NaCl
    - Deionized or distilled water

Perform Photocalibration

1. Follow instructions in OxyScan™ 340 nm Photocalibrator package insert.
2. Perform photocalibration at 340 nm.

Pump Preparation

1. Insert reagents into the proper wells:

t-BOOH Working Dilution	Pump 1
Empty	Pump 2
Reconstituted NADPH Reagent	Pump 3
Empty	Pump 4

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2. Insert proper tubing into the reagent bottles.
3. Lift the reagent arm and place the waste cup beneath the tubing exit.
4. Ensure the pump cover is in place. Press PUMP ENGAGE/RELEASE to engage the pumps. Press PRIME. Select PRIME ALL.
5. When the screen changes to the step pumps, press each pump number to pipet 100 mL of fluid to confirm tubing is primed.
6. Press ESC.

Instrument Preparation

1. Press UTILITY and scroll down to REPLICATE SELECTION.
2. Choose number of replicates (3 is recommended).
3. If running an assay calibration curve with the samples, perform steps 4 and 5.
4. Scroll down to the ASSAY CAL screen and choose the CONC.
5. Choose GPx, enter the calibrator concentration values for the OxyScan™ GPx-340 Calibrator Kit from its package insert.
6. Return to the main menu.

Cuvette/Carousel Preparation

1. Pipet 200 mL deionized water into the first two positions of the carousel to serve as blanks.
2. If running an assay calibration curve with the samples, perform step 3.
3. Starting with position 3, pipet 50 mL of each calibrator level into the appropriate number of wells equivalent to the replicates to run (Instrument Preparation step 2).
4. Pipet 50 mL of each diluted sample in the replicates specified into the following wells .
5. Insert cuvette(s) into the carousel, starting with the first blank in position 1.

Assay Run

1. Press ASSAYS and select YES or NO for assay calibrators during run.
2. Select GPx.
3. Enter the number of samples to test, not total number of replicates. (This number does not include calibrators or blanks).
4. Press SAMPLE ID to enter numerical IDs for the samples including calibrators. (Press ESC to exit the menu).
5. Ensure cover is down.
6. Press RUN.

Calculation of Results

1. The OxyScan™ printout reports the concentration of the sample placed in the cuvette.
2. This result may be corrected for sample preparation dilutions.

BIBLIOGRAPHY

1. Ursini, F., et al (1995) Meth. in Enzymol. 252: 38-53.
2. D.E. Paglia and W.N. Valentine (1967) J. Lab. Clin. Med. 70: 158-169.

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SUMMARY

Catalog #	51017
Calibrator Requirement:	Also requires calibrator # 52017
Photo Calibrator Requirement:	Also requires OxyScan™ 340 nm photocalibrator
Specimen Requirements:	Purified enzymes or clear supernatant from cell lysate or tissue homogenate
Kit Contents:	(suitable for 100 triplicate determinations) NADPH Reagent (lyophilized) NADPH GSH GSSG tert-Butyl hydroperoxide Buffer
Sensitivity:	6mU/mL Final concentration in the assay.
Specificity:	Specific for cellular glutathione peroxidase activity.

OXIS Health Products, Inc. 800-547-3686

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